Docket No.: 22122-00009-US1
(PATENT)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of:
Florian V. Mulbe et al.

Application No.: 10/729,830 Confirmation No.: 8653

Filed: December 5, 2003 Art Unit: 1636

For: PHARMACEUTICAL COMPOSITION CONTAINING A STABILISED mRNA OPTIMISED FOR TRANSLATION IN ITS CODING REGIONS Examiner: J. A. Dunston

SECOND DECLARATION OF DR. INGMAR HOERR

Dr. Ingmar Hoerr deposes and states as follows:

1. I am an inventor named on the above-captioned patent application and I am affiliated with the assignee of the application. I provided a previous declaration in connection with this application, which was dated October 10, 2006. My scientific background and technical qualifications are provided in that prior declaration.

2. Additional experiments have been performed under my direction and control, the results of which are reported in this second declaration.

3. The experiments reported in this second declaration were directed towards investigating the anti-tumour response obtained upon vaccination of an animal by injection of either wild-type mRNA encoding a human tumour antigen, or, for comparison, an mRNA encoding the same tumour antigen but which had been modified to contain a higher content of GC relative to wild type, in accordance with the disclosure of this patent application.

4. Two types of experiments were performed:

a. Using tumour challenge experiments, we investigated whether tumour growth could be reduced by vaccination with GC-enriched mRNA in comparison to vaccination using the respective wild-type mRNA coding for the human tumour antigens Survivin, GP100 and TRP-2; and

b. Using ELISPOT experiments, we investigated whether vaccination with GC-enriched mRNA coding for the tumour antigens MAGE-A2, MAGE-C2 and STEAP leads to the induction of more tumour antigen specific cytotoxic T-cells as compared to vaccination with the corresponding wild type mRNAs.

5. The results of our experiments are shown in the graphs provided below. The experimental methods are described in the text which follows. Attached to this declaration are the nucleotide sequences of the mRNAs which were used in the experiments.

6. To summarize the results of the experiments:

a. In the tumour challenge experiments, it was demonstrated that tumour growth is reduced more efficiently by vaccination with GC-enriched mRNA, as compared with the corresponding wild type mRNA coding for the human tumour antigens Survivin, GP100 and TRP-2. The tumour model used in these experiments is an accepted model for determining vaccine activity to inhibit growth of human tumours.

b. The ELISPOT experiments showed that vaccination with GC-enriched mRNA coding for the tumour antigens MAGE-A2, MAGE-C2 and STEAP leads to the induction of more tumour antigen specific cytotoxic T- cells than does vaccination with the corresponding wild-type mRNA. These results are significant, since it has been shown in recent years that induction of tumour antigen-specific cytotoxic T-cells is necessary for induction of immunity against tumour cells *in vivo*. The ELISPOT data confirms that GC-

enriched mRNA coding for a human tumour antigen is more potent for tumour vaccination than the corresponding wild type mRNA.

7. The ELISPOT assay is described in Janeway, Charles A.; Travers, Paul; Walport, Mark; Shlomchik, Mark; Immunobiology; New York and London: Garland Science; 2001, and is a modification of the ELISA antigen-capture assay. The ELISPOT assay is recognized to be a useful tool for measuring the frequency of T-cell responses. In the method, populations of T cells are stimulated with the antigen of interest (in this case, re-stimulation with an antigen-specific peptide library, as described *infra*), and are then allowed to settle onto a plastic plate coated with antibodies to the cytokine which is to be assayed (here, INF-gamma). If an activated T cell is secreting the cytokine, it is captured by the antibody on the plastic plate. After an appropriate period of time, the cells are removed, and a second antibody which is reactive to the cytokine is added to the plate, which reveals a circle of bound cytokine surrounding the position of each activated T cell. By counting each spot, and by knowing the number of T cells originally added to the plate, one may readily calculate the frequency of T cells which are secreting the cytokine. In this manner, we used the ELISPOT assay to quantify the number of antigen specific cytotoxic T cells which were present in the experimental animals following vaccination with mRNA encoding for several tumour antigens.

8. The murine B16 melanoma tumour model has been known and used for several years, having been described by Fidler, I.J., Selection of successive tumour lines for metastasis. Nat. New Biol., 242:148-149, 1973. B16 melanoma cells were isolated from C57BL/6 mice and provide a syngenic tumour model for evaluating the efficacy of anti-tumour vaccines. Since there is no *in vivo* human model available for use by workers in this field, this model is an important and art-recognized model for evaluating human tumour vaccination strategies.

9. Recombinant plasmid DNA was linearized and subsequently *in vitro* transcribed using T7 RNA polymerase. The DNA template was then degraded by DNAseI digestion. The mRNA was recovered by LiCl precipitation and further

cleaned by HPLC extraction (Pure Messenger®, CureVac GmbH, Tübingen, Germany).

10. The animals used were C57BL/6 mice, in groups of 5 mice per group. The tumour cells used were B16F10 melanoma cells. Immunization was performed with 20 µg mRNA (10 µg per ear) complexed wit protamine (RNA:protamine (8:1)) which was injected intradermally in the mice 8 times over a period of 3 weeks.

11. For the tumor challenge experiments, the mice were divided into groups which received vaccination with the following mRNAs:

Groups:

- Survivin wt
- Survivin GC
- GP100 wt
- GP 100 GC
- TRP2 wt
- TRP2 GC

One week after the last immunization, 4×10^5 B16 melanoma cells were injected subcutaneously in the mice. For the next 10-17 days tumour volume was determined.

Figure 4 shows the results of the tumour challenge experiment with B16 melanoma cells in C57BL/6 mice, one week after the last vaccination with wild-type mRNA (Survivin wt) or GC-enriched mRNA encoding Survivin (Survivin GC). Tumour cells were implanted and tumour growth was observed for 10 days.

Figure 5 shows a tumour challenge experiment with B16 melanoma cells in C57BL/6 mice, one week after the last vaccination with wild-type mRNA (GP100 wt) or GC-enriched mRNA (GP100 GC). Tumour cells were implanted and tumour growth was observed for 17 days.

Figure 6 shows a tumour challenge experiment with B16 melanoma cells in C57BL/6 mice, one week after the last vaccination with wild type

mRNA (TRP2 wt) or GC-enriched mRNA (TRP2 GC). Tumour cells were implanted and tumour growth was observed for 17 days.

12. The results depicted in Figures 4-6 are, in my opinion, predictive of the utility of GC-enriched mRNA encoding a human tumor antigen to treat tumours in a human patient. B16 melanoma cells express endogenously the tumour antigens Survivin, GP100 and TRP2. Accordingly, those antigens were chosen for the vaccination experiments. Mice receiving prophylactic vaccination with wild type or GC-enriched mRNA coding for these antigens were compared, and the results reflect that mice which were vaccinated with GC-enriched mRNA showed a reduced increase in tumour growth as compared to the mice vaccinated with wild type mRNA. These results suggest that vaccination with GC-enriched mRNA coding for a human tumour antigen will be more effective for vaccination than wild type mRNA.

13. For the ELISPOT experiments, the mice were divided into groups which received vaccination with the following mRNAs:

Groups:

- MAGE-A2 wt
- MAGE-A2 GC
- MAGE-C2 wt
- MAGE-C2 GC
- STEAP wt
- STEAP GC

Two weeks after the last vaccination, the mice were sacrificed, the spleens were removed, and the splenocytes were isolated. For re-stimulation, 3×10^7 splenocytes were incubated for 5 days with an antigen-specific peptide library (1 µg/ml per peptide) in presence of IL-2 (30 U/ml). To determine an antigen-specific cellular immune response, IFNγ secretion was measured after re-stimulation with peptide. For detection of IFNγ a coat multiscreen plate (Millipore) was incubated overnight with coating buffer (0,1 M Carbonat-Bicarbonat Buffer pH 9,6, 10,59 g/l Na_2CO_3, 8,4g/l $NaHCO_3$) comprising

antibody against IFNγ (BD Pharmingen, Heidelberg, Germany). After coating, effector cells (5×10^5/well) were incubated with the antigen-specific peptide library, an unspecific control peptide, DMSO or medium as control for 24h in the plate. Subsequently the plate was washed with 1×PBS and incubated with a biotin-coupled secondary antibody. After washing with 1×PBS/0,05% Tween-20 the substrate (5-Bromo-4-Cloro-3-Indolyl Phosphate/Nitro Blue Tetrazolium Liquid Substrate System from Sigma Aldrich, Taufkirchen, Germany) was added to the plate and the conversion of the substrate could be detected visually.

Figure1 shows the results of the ELISPOT assay measuring the IFNγ secretion of splenocytes of mice which were vaccinated respectively with wild type mRNA (MAGE-A2 wt) or GC-enriched mRNA (MAGE-A2 GC) coding for MAGE-A2. The secretion of IFNγ by splenocytes was measured after re-stimulation with a MAGE-A2 specific peptide library (antigen-specific peptide library), DMSO, an unspecific control peptide library (control peptide) or medium as control.

Figure 2 shows the results of the ELISPOT assay measuring the IFNγ secretion of splenocytes of mice which were vaccinated respectively with wild type mRNA (MAGE-C2 wt) or GC-enriched mRNA (MAGE-C2 GC) coding for MAGE-C2. The secretion of IFNγ of splenocytes was measured after re-stimulation with a MAGE-C2 specific peptide library (antigen-specific peptide library), DMSO, an unspecific control peptide library (control peptide) or medium as control.

Figure 3 shows the results of the ELISPOT assay measuring the IFNγ secretion of splenocytes of mice which were vaccinated respectively with wild type mRNA (STEAP wt) or GC-enriched mRNA (STEAP GC) coding for STEAP. The secretion of IFNγ of splenocytes was measured after re-stimulation with a STEAP specific peptide library (antigen-specific peptide library), DMSO, an unspecific control peptide library (control peptide) or medium as control.

14. The results presented in Figures1-3 illustrate that vaccination with GC-enriched mRNA coding for a tumour antigen initiates a stronger anti-tumor cytotoxic T cell immune response than does vaccination with wild type mRNA. As persons working in this art would appreciate, a stronger cytotoxic T cell immune response is likely to correlate with improved anti-tumour efficacy *in vivo*.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of this application of any patent issued thereon.

Date: 14-Oct. 2008

Dr. Ingmar Hoerr

Fig. 1

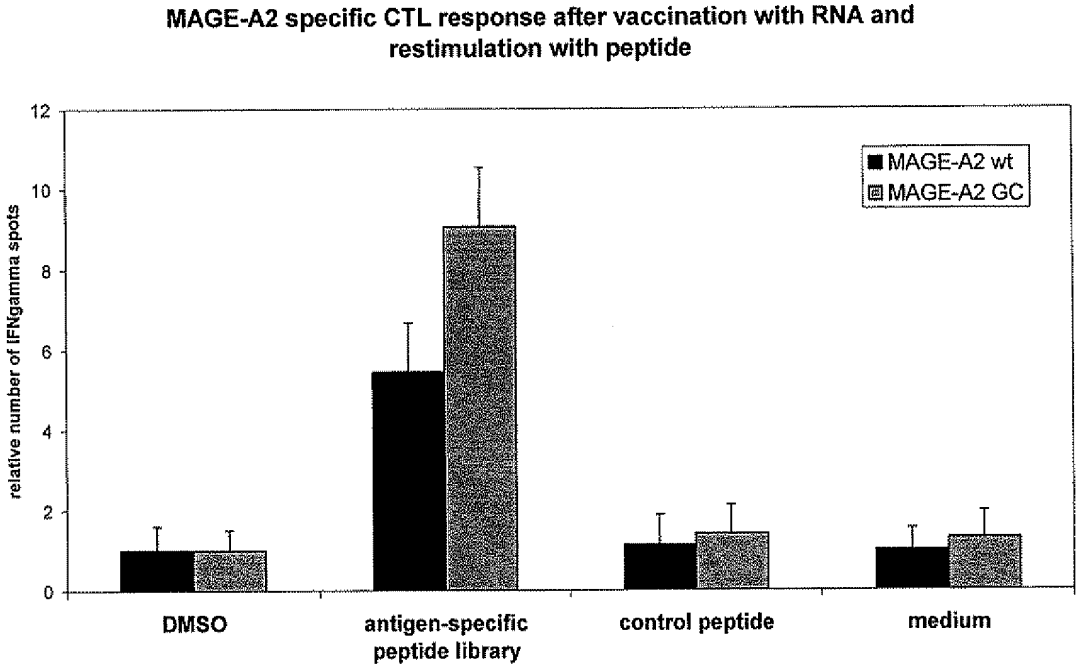

MAGE-A2 specific CTL response after vaccination with RNA and restimulation with peptide
relative number of IFNgamma spots
12
10
8
6
4
2
0
MAGE-A2 wt
MAGE-A2 GC
DMSO
antigen-specific peptide library
control peptide
medium

Fig. 2

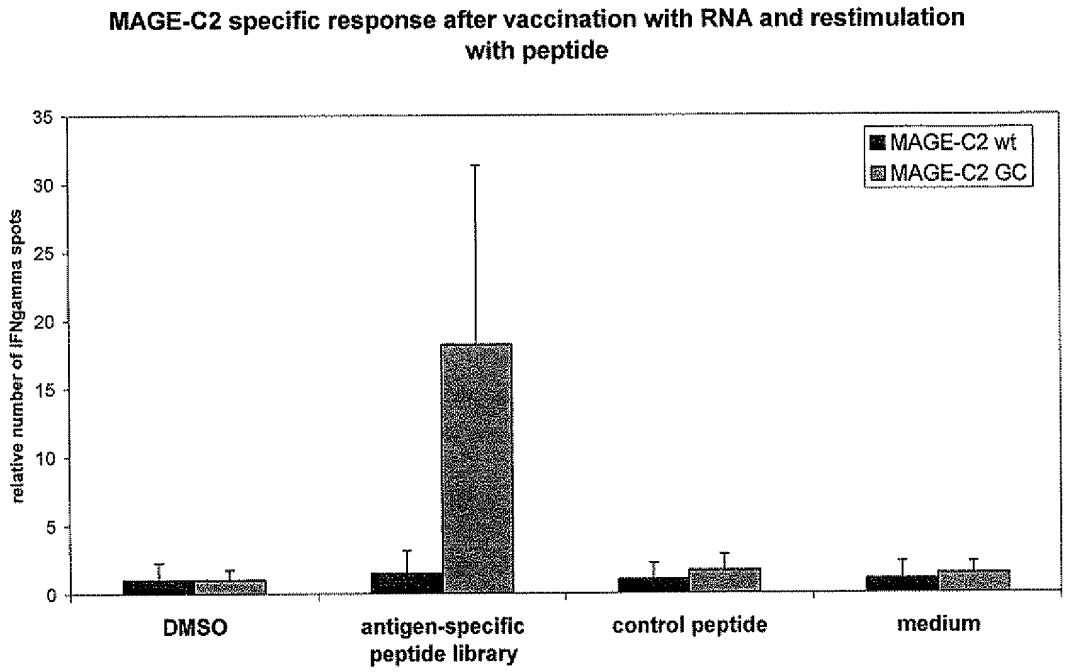

MAGE-C2 specific response after vaccination with RNA and restimulation with peptide
relative number of IFNgamma spots
35
30
25
20
15
10
5
0
MAGE-C2 wt
MAGE-C2 GC
DMSO
antigen-specific peptide library
control peptide
medium

Fig. 3

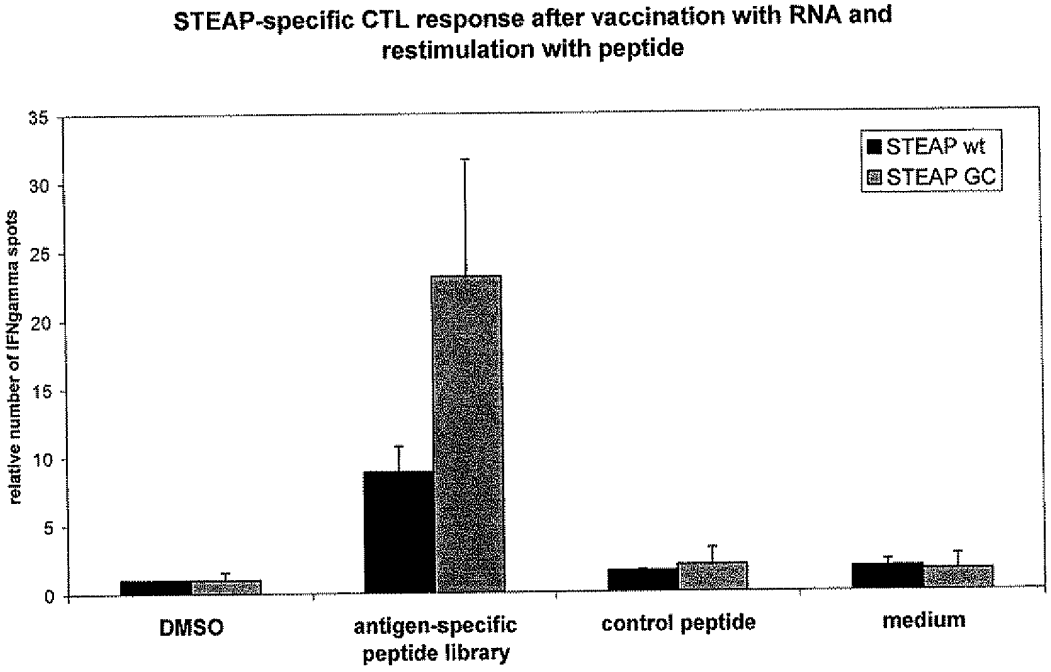

STEAP-specific CTL response after vaccination with RNA and restimulation with peptide
relative number of IFNgamma spots
35
30
25
20
15
10
5
0
STEAP wt
STEAP GC
DMSO
antigen-specific peptide library
control peptide
medium

Fig. 4

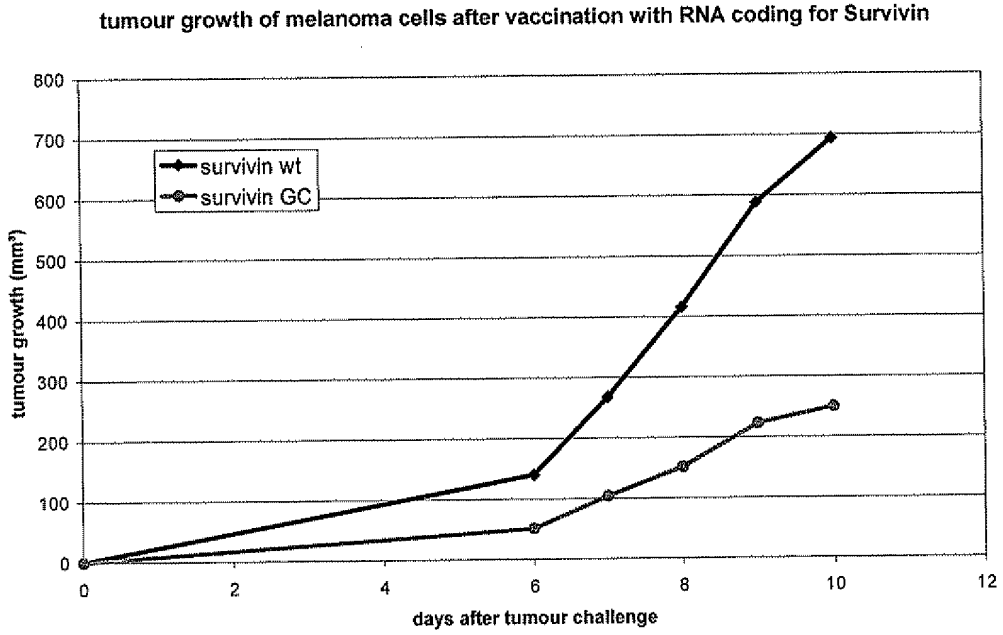

tumour growth of melanoma cells after vaccination with RNA coding for Survivin
tumour growth (mm³)
800
700
600
500
400
300
200
100
0
survivin wt
survivin GC
0
2
4
6
8
10
12
days after tumour challenge

Fig. 5

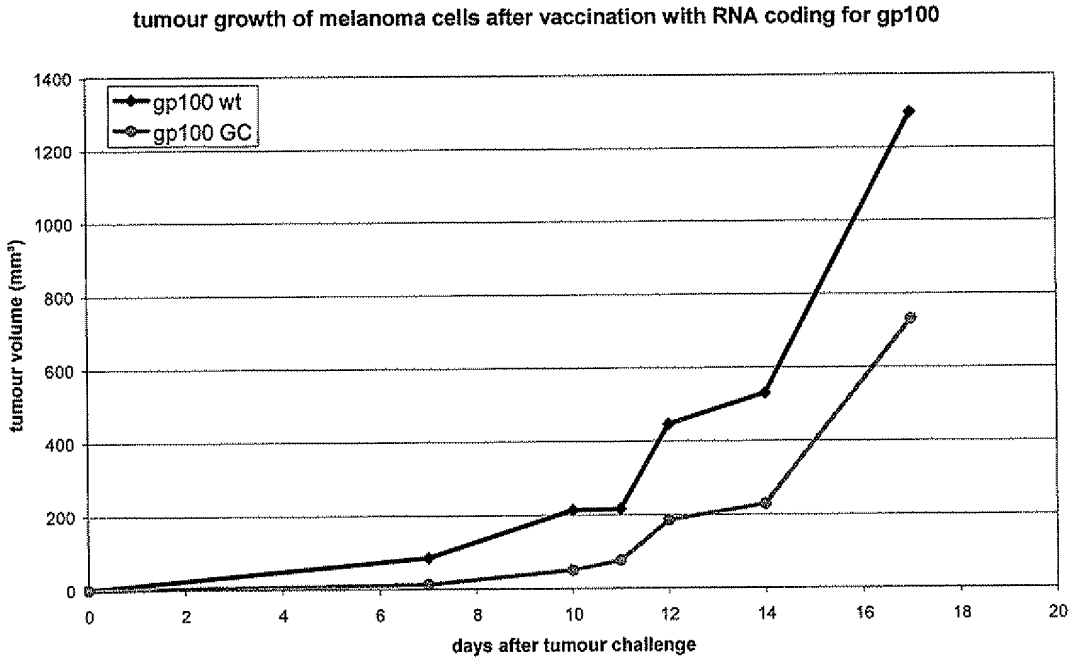
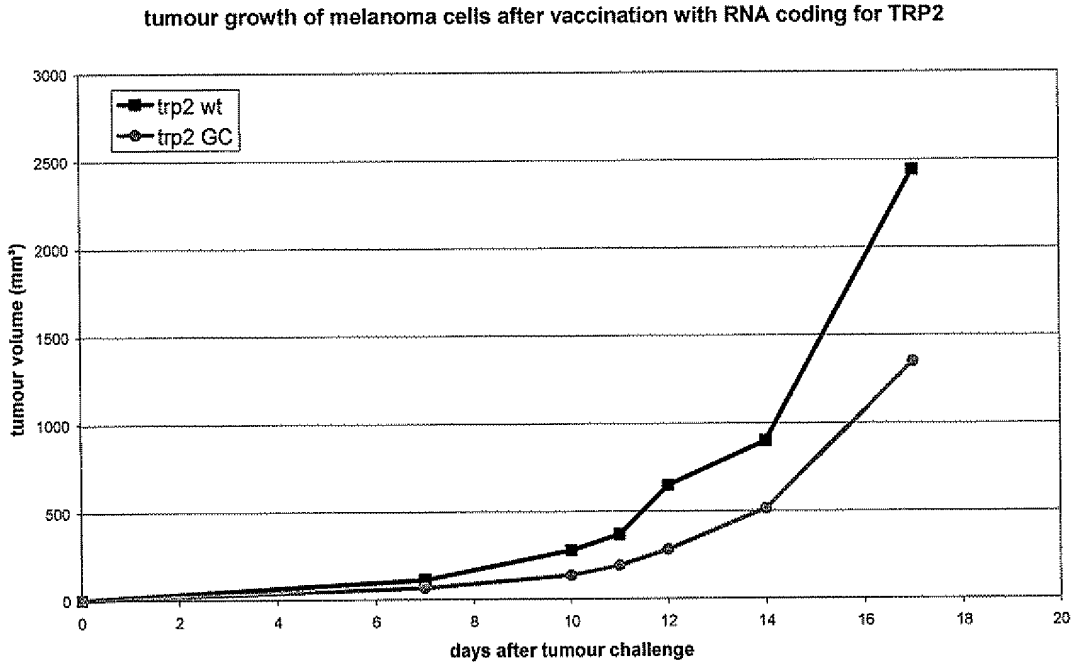

tumour growth of melanoma cells after vaccination with RNA coding for gp100
gp100 wt
gp100 GC
tumour volume (mm³)
days after tumour challenge
0
200
400
600
800
1000
1200
1400
0
2
4
6
8
10
12
14
16
18
20

Fig. 6


tumour growth of melanoma cells after vaccination with RNA coding for TRP2
trp2 wt
trp2 GC
tumour volume (mm³)
days after tumour challenge
0
500
1000
1500
2000
2500
3000
0
2
4
6
8
10
12
14
16
18
20

Sequences:

MRNA HsTRP2(wt)-ag-A70

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MRNA HsTRP2(GC)-muag-A70-C30

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MRNA HsSILV-isoCRA_c(wt)-ag-A70 (SILV = GP100)
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MRNA HsSILV(GC)-muag-A70-C30

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MRNA HsSurvivin(wt)-ag-A70

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TGAGGAAACTGCGAAGAAAGTGCGCCGTGCCATCGAGCAGCTGGCTGCCATGGATTGAccACTA
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MRNA HsSurvivin(GC)-muag-A70-C30

GGGAGAAAGCTTaccATGGGCGCCCCCACCCTGCCGCCGGCCTGGCAGCCGTTCCTCAAGGACC
ACCGCATCTCGACCTTCAAGAACTGGCCGTTCCTGGAGGGCTGCGCGTGCACCCCGGAGCGGA
TGGCCGAGGCCGGCTTCATCCACTGCCCCACCGAGAACGAGCCGGACCTGGCCCAGTGCTTCT
TCTGCTTCAAGGAGCTGGAGGGCTGGGAGCCGGACGACGACCCGATCGAGGAGCACAAGAAGC
ACAGCAGCGGCTGCGCCTTCCTGAGCGTGAAGAAGCAGTTCGAGGAGCTGACGCTCGGGGAGT
TCCTGAAGCTGGACCGGGAGCGGGCCAAGAACAAGATCGCGAAGGAGACCAACAACAAGAAGA
AGGAGTTCGAGGAGACCGCCAAGAAGGTGCGGCGGGCCATCGAGCAGCTGGCCGCCATGGACt
gaccACTAGTTATAAGACTGACTAGCCCGATGGGCCTCCCAACGGGCCCTCCTCCCCTCCTTGCA
CCGAGATTAATAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA
AAAAAAATATTCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCTCTAGACAATTGGAATT

MRNA HsMageA2(wt)-ag-A70

GGGAGACAAGCTTaccatgcctcttgagcagaggagtcagcactgcaagcctgaagaaggccttgaggcccgaggagaggccctg
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TCCTCCCCTCCTTGCACCAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA
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MRNA HsMageA2(GC)-muag-A70-C30
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GAGGCCCGCGGGGAGGCCCTGGGCCTGGTGGGGGCGCAGGCCCCCGCCACCGAGGAGCAGC
AGACCGCCTCCAGCTCCAGCACGCTCGTCGAGGTGACCCTGGGCGAGGTGCCCGCCGCGGACT
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TGCTGGAGGTGTTCGAGGGCCGGGAGGACTCCGTGTTCGCCCACCCCCGCAAGCTGCTCATGC
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CCTCAAGATCGGGGGCGAGCCCCACATCAGCTACCCGCCGCTGCACGAGCGGGCCCTGCGCGA
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GGAATT

MRNA HsMageC2(wt)-ag-A70
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MRNA HsMageA2(GC)-muag-A70-C30
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MRNA HsSTEAP(wt)-ag-A70
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MRNA HsSTEAP(GC)-muag-A70-C30

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